A Dominant Mutation in the *Chlamydomonas reinhardtii* Nuclear Gene *SIM30* Suppresses Translational Defects Caused by Initiation Codon Mutations in Chloroplast Genes

Xuemei Chen,[1] Clare L. Simpson, Karen L. Kindle* and David B. Stern

*Boyce Thompson Institute for Plant Research and *Plant Science Center, Cornell University, Ithaca, New York 14853*

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ABSTRACT

A suppressor of a translation initiation defect caused by an AUG to AUU mutation in the *Chlamydomonas reinhardtii* chloroplast *petD* gene was isolated, defining a nuclear locus that we have named *SIM30*. A dominant mutant allele at this locus, *sim30-1d*, was found to increase the translation initiation rate of the mutant *petD* mRNA. *sim30-1d* was also able to suppress the translational defect caused by an AUG to AUC mutation in the *petD* gene, and an AUG to AUU mutation in the chloroplast *petA* gene. We therefore suggest that the *SIM30* gene may encode a general chloroplast translation factor. The ability of *sim30-1d* to suppress the *petD* AUG to AUU mutation is diminished in the presence of one or more antibiotic resistance markers located within the 16S and 23S rRNAs, suggesting that the activity of the *sim30-1d* gene product in translation initiation may involve interaction with ribosomal subunits.

TRANSLATION initiation generally requires an AUG codon placed in a context where it can be recognized efficiently by ribosomes and their associated factors. In chloroplasts, AUG is nearly universally found at the N-terminus of coding regions, with GUG as a rare exception (ROCHAIX *et al.* 1989; TURMEL *et al.* 1993). Similarly, 91% of *Escherichia coli* initiation codons are AUG, with most of the remainder being GUG (HERSHEY 1987).

In contrast to the situation in *E. coli*, however, there is no clear sequence consensus in the region surrounding chloroplast translation initiation codons. In fact, only 50% of 196 chloroplast genes examined contain putative Shine-Dalgarno sequences in their 5′ untranslated regions (UTRs) within 12 nucleotides of the initiation codon (BONHAM-SMITH and BOURQUE 1989). Although mutations in a Shine-Dalgarno-like sequence in the Chlamydomonas chloroplast *psbA* gene may disable translation (MAYFIELD *et al.* 1994), mutations in a similar sequence in the *petD* gene had no effect on translation initiation (SAKAMOTO *et al.* 1994). It is therefore likely that additional mechanisms for initiation codon recognition exist in chloroplasts. Genetic and molecular evidence suggests that sequence elements located in the 5′ UTR interact with nuclear-encoded gene-specific translation factors to effect translation initiation (reviewed in ROCHAIX 1992). Genes encoding chloroplast proteins with sequence similarity to bacterial IF-1 (*e.g.*, SIJBEN-MULLER *et al.* 1986; FUKUZAWA *et al.* 1988) and IF-3 (LIN *et al.* 1994) have been identified in the chloroplast and nuclear genomes; IF-2 is probably also encoded in the nucleus (KRAUS and SPREMULLI 1988). It is possible that general translation factors in addition to homologues of IF-1, IF-2 and IF-3 exist in chloroplasts and facilitate initiation codon recognition.

As a first step in identifying chloroplast translation factors that function in initiation codon recognition we have taken a molecular genetic approach, using *Chlamydomonas reinhardtii* as a model system. We previously introduced initiation codon mutations into two Chlamydomonas chloroplast genes, *petA* and *petD* (CHEN *et al.* 1993, 1995). *petA* and *petD* encode cytochrome f and subunit IV of the cytochrome b_6/f complex, respectively; this complex is composed of at least seven proteins (LEMAIRE *et al.* 1986; CRAMER *et al.* 1996) and is an essential component of the photosynthetic electron transport chain. Consequently, null or near-null mutations in either the *petA* or *petD* gene cause a photosynthetic growth defect (CHEN *et al.* 1993; KURAS and WOLLMAN 1994). When the *petD* AUG initiation codon was changed to either AUU (strain iniD2) or AUC (strain iniD1), subunit IV translation decreased to 10% of the wild-type level, resulting in a reduced photosynthetic growth rate at 24°. At 35°, these strains failed to grow photosynthetically (CHEN *et al.* 1993). Mutating the *petA* AUG initiation codon to AUU, ACG, ACC, ACU or UUC reduced the translation of cytochrome f to 10–20%, 2–5%, 1%, <1% and 0% of the wild-type level, respectively (CHEN *et al.* 1995).

We have isolated a suppressor of the temperature-sensitive nonphotosynthetic growth phenotype of the iniD2 (*petD*-AUU) mutant. Here, we report the characterization of this dominant nuclear-encoded allele,

Corresponding author: David B. Stern, Boyce Thompson Institute for Plant Research, Tower Road, Cornell University, Ithaca, NY 14853. E-mail: ds28@cornell.edu

[1] *Present address:* Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125.

which suppresses chloroplast initiation codon mutations in both *petA* and *petD*. Studies described below suggest that this nuclear gene may encode a general chloroplast translation factor that interacts with the ribosome.

MATERIALS AND METHODS

Plasmids: The plasmid p-183 was obtained from the Chlamydomonas Genetic Stock Center at Duke University and contains the 7-kb *Bam*HI 11/12 fragment, which includes 16S rDNA and the 5′ end of 23S rDNA. It contains two mutations in the 16S rDNA (*spr-u-1-6-2*, and *sr-u-2-60*), which confer resistance to spectinomycin and streptomycin, respectively; and a mutation in 23S rDNA (*er-u-37*), which confers resistance to erythromycin (HARRIS *et al.* 1989). The pBam7 plasmid contains the *Bam*HI 7 fragment of the Chlamydomonas chloroplast genome and includes the *petA*, *petD* and *trnR* genes (HARRIS 1989).

Strains, culture conditions and chloroplast transformation: Strains were grown in HSA or YA medium (HARRIS 1989) under continuous medium intensity (5–8 W/m^2) or dim (~1.3 W/m^2) light. M-N medium (HARRIS 1989) was used to induce gametogenesis. The strains used in this research are listed in Table 1. P17 was created by transforming CC373, which contains a deletion in the *atpB* gene in the chloroplast genome, with the wild-type *atpB* gene (STERN *et al.* 1991). iniD2 and iniD1 were derived from P17 by transformation and contain the *spr-u-1-6-2* mutation in 16S rDNA, which confers spectinomycin resistance, as well as AUG to AUU or AUC mutations at the initiation codon position of the chloroplast *petD* gene (CHEN *et al.* 1993). The A-AUU, A-ACG and A-ACC strains contain the same spectinomycin-resistance mutation and AUG to AUU, ACG or ACC mutations in the initiation codon position of the chloroplast *petA* gene (CHEN *et al.* 1995). CC1861 and CC1930 were obtained from the Chlamydomonas Genetic Stock Center at Duke University. The rest of the strains listed in Table 1 were created in this study. The AUG initiation codon was introduced into the strain S30 to replace the AUU mutation through chloroplast cotransformation as described by CHEN *et al.* (1993), using the selectable markers contained within p-183.

Genetic crosses: Strains were streaked onto YA plates and allowed to grow for 5–8 days under dim light while covered loosely by aluminum foil. Cells from each mt^- and mt^+ strain were resuspended in 3 ml of M-N medium at ~10^6 cells/ml and kept under medium intensity light for 10–11 hr to induce gametogenesis. Mating and tetrad analysis were carried out as described (HARRIS 1989).

Construction of diploids: Gametogenesis and mating were carried out as described (HARRIS 1989). Strains containing complementing mutations in the gene that encodes argininosuccinate lyase (*arg7-8* and *arg7*) were allowed to mate for 2 hr at room temperature under medium intensity light. Aliquots of the mating mixture were spread onto plates lacking arginine (HSA) and incubated under dim light for several weeks to select diploid colonies. Since the chloroplast genome in these diploids was initially heteroplasmic, they were streaked to obtain single colonies. Single colonies obtained this way were homoplasmic and contained the chloroplast genotype of either parent, as determined using the PCR-based analysis shown in Figure 2.

DNA isolation and PCR: DNA was isolated and PCR amplification was performed as described (CHEN *et al.* 1993). The primers WS6 and WS4 used in this study were as described (SAKAMOTO *et al.* 1993, 1994).

Total protein preparation and immunoblotting: Protein preparation and immunoblotting were carried out as described (CHEN *et al.* 1993). Antibodies against spinach CF1-β were kindly provided by R. MCCARTY (Johns Hopkins University) and used at a dilution of 1:100,000. Antibodies against Chlamydomonas subunit IV (CHEN *et al.* 1993) and cytochrome f (CHEN *et al.* 1995) were used at dilutions of 1:1000 and 1:5000, respectively.

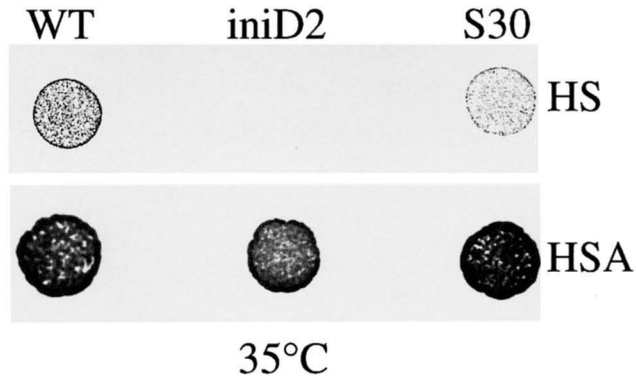

FIGURE 1.—The *sim30-1d* mutation restores photosynthetic growth at 35°. Wild-type (WT), iniD2 and S30 cells were grown in HSA medium at 24° to early log phase, diluted 10-fold with HS, and spotted onto HS or HSA plates. The plates were incubated at 35° under medium intensity light (5–8 W/m^2) for 5 days.

RNA isolation and RNA filter hybridizations: Cells were grown to mid-log phase in HSA medium at room temperature. RNA isolation and filter hybridizations were carried out as described (STERN *et al.* 1991). The *petD* probe was a PCR fragment amplified from pBam7 using primers WS4 and WS6; the *atpB* probe was a PCR fragment amplified from p17 (STERN *et al.* 1991) using primers DBS2 and NS1b (CHEN *et al.* 1995). RNA was quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

***In vivo* pulse labeling and pulse-chase experiments:** To measure chloroplast protein synthesis rates, cells were labeled for 5 min with ^{14}C-acetate (DELEPELAIRE 1983) or ^{35}S-Na_2SO_4 (SAKAMOTO *et al.* 1994) in the presence of 8 μg/ml cycloheximide as an inhibitor of cytosolic translation.

The procedure used for the pulse-chase experiments was adapted from that described by KURAS and WOLLMAN (1994). Cells were first labeled for 5 min with ^{35}S-Na_2SO_4 in the presence of 8 μg/ml cycloheximide as described (SAKAMOTO *et al.* 1994), and then chased by addition of 10 vol of HSA medium containing 40 mM Na_2SO_4. Aliquots were removed after 0, 30, 60, 120, 240 and 480 min, and the chase was stopped by addition of 1 vol of chilled 80 mM Na_2SO_4. Proteins were prepared and electrophoresed in 15% SDS-polyacrylamide gels.

RESULTS

***sim30-1d* suppresses an initiation codon mutation in the chloroplast *petD* gene:** We isolated a phenotypic revertant of the temperature-sensitive photosynthetic growth phenotype in iniD2, which is caused by reduced translation of subunit IV from *petD*-AUU mRNA. This strain, designated S30, was isolated as a photosynthetic colony from a lawn of nonphotosynthetic iniD2 cells on HS plates at 35°. Figure 1 shows the growth of P17 (WT), iniD2 ([*D-AUU*]) and S30 (*sim30-1d* [*D-AUU*]) on HS and HSA (acetate-containing) plates at 35°.

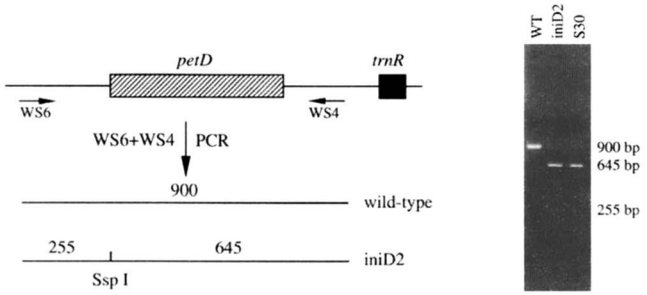

FIGURE 2.—S30 still contains the *D-AUU* mutation. PCR products amplified from total DNA using primers WS6 and WS4 were digested with *Ssp*I. The presence of the *Ssp*I site indicates the presence of the AUU mutation, as shown in the left panel.

iniD2 failed to grow photosynthetically at this temperature, whereas S30 showed moderate photosynthetic growth when compared with the wild-type strain. To determine whether the AUU mutation was still present in S30, we PCR-amplified a 900-bp fragment encompassing the initiation codon from P17 (WT), iniD2 and S30, and digested these fragments with *Ssp*I; an *Ssp*I site had been created by the AUU mutation. Figure 2 shows that the 900-bp fragments from both iniD2 and S30 were digested into fragments of 255 and 645 bp, whereas the fragment from P17 (referred to below as WT) remained undigested. We therefore concluded that S30 is not a revertant of the initiation codon mutation, but instead contains a second-site mutation.

As described below, S30 carries a dominant mutation at a nuclear locus, which we have termed *SIM30* (suppressor of initiation codon mutation). The mutant allele is designated *sim30-1d* according to the guidelines recently adopted by the Chlamydomonas community (DUTCHER 1995). The chloroplast genotype is displayed in brackets following the nuclear genotype.

***sim30-1d* increases the accumulation of subunit IV from the *petD-AUU* gene:** We quantified the accumulation of subunit IV in S30 by immunoblotting. WT, iniD2 and S30 were grown in acetate-containing medium (HSA) to late log phase, and total proteins were separated by electrophoresis, blotted to nitrocellulose and reacted with antibodies specific for subunit IV or CF1-β. A dilution series of proteins from WT was used to calibrate the relative levels of subunit IV accumulation in iniD2 and S30. Figure 3A shows that iniD2 accumulated ~10% as much subunit IV as the wild-type strain. In S30, however, subunit IV accumulated to ~50% of the level in the wild-type strain, suggesting that *sim30-1d* increases the accumulation of subunit IV approximately fivefold. This quantification of subunit IV accumulation was confirmed by using a Phosphorimager to scan immunoblots probed with ^{125}I-protein A (data not shown). Increased accumulation of subunit IV in S30 is consistent with the more robust photosynthetic growth of S30 relative to iniD2 on HS plates at 24° (data not shown). S30 cells grown in HSA at 35° also exhibited increased

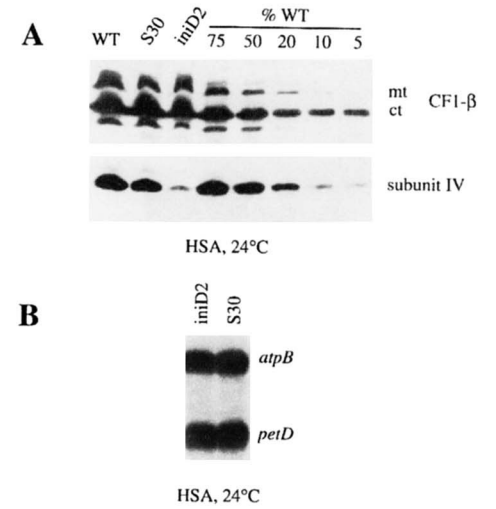

FIGURE 3.—*sim30-1d* increases the accumulation of subunit IV from the *petD-AUU* gene without increasing *petD* mRNA. (A) Cells were grown in HSA at 24°, and total proteins were isolated and analyzed by immunoblotting using antibodies raised against subunit IV and CF1-β. A dilution series of wild-type proteins was included to estimate the amount of subunit IV in S30 and iniD2. Both the mitochondrial (mt) and chloroplast (ct) forms of CF1-β are detected with this antibody. (B) Total RNA was isolated from the indicated strains, and filter blots were probed with the *petD* and *atpB* coding regions.

accumulation of subunit IV (data not shown), which is consistent with the photosynthetic growth phenotype at 35° (Figure 1). The observation that the *sim30-1d* mutation increased subunit IV accumulation at 24° facilitated its further characterization, and all further experiments described in this paper were carried out with cells grown at 24°.

***sim30-1d* increases the subunit IV translation rate but has no effect on petD gene copy number or mRNA accumulation:** The effect of *sim30-1d,* which is to increase the accumulation of subunit IV from the *petD-AUU* gene, could in theory be achieved by one or more different mechanisms. The suppressor could act on chloroplast DNA replication or transcription to increase either the *petD* gene copy number or its transcription rate. It could also act posttranscriptionally at the level of mRNA stability, translation initiation, or protein stability.

Although we considered amplification of the *petD* gene or the entire chloroplast genome to be an unlikely suppression mechanism, we have previously shown that under certain conditions such amplification can occur (KINDLE *et al.* 1994). We compared the chloroplast DNA copy number in iniD2 and S30 by isolating total DNA, and carrying out filter hybridizations with a nuclear probe (the nitrate reductase gene) and a chloroplast probe (the *petD* gene). By quantifying the hybridization signals with the chloroplast probe in the two strains and using the nuclear probe as an internal standard, we concluded that *sim30-1d* does not affect chloroplast DNA copy number (data not shown). To determine

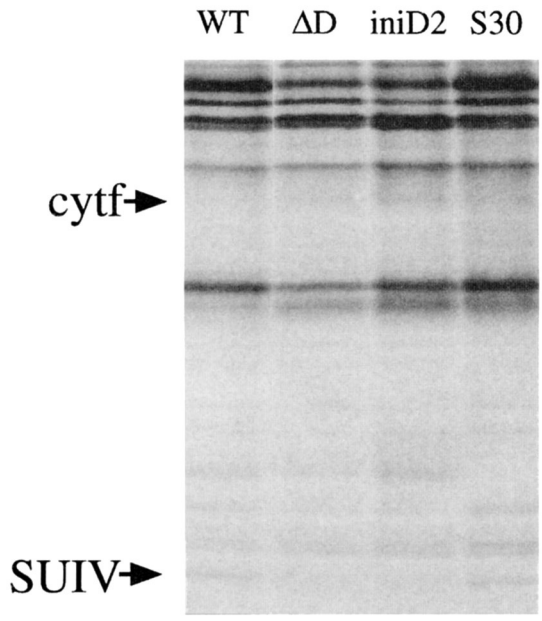

FIGURE 4.—Subunit IV synthesis is increased in S30 cells. Chloroplast proteins were pulse-labeled for 5 min with ^{14}C-acetate as described in MATERIALS AND METHODS. Total proteins were analyzed by electrophoresis in a 12–18% SDS-polyacrylamide gel containing urea. The gel was exposed to a Phosphorimager screen for 3 weeks.

whether *sim30-1d* increases *petD* mRNA accumulation, we performed RNA filter hybridizations. Using *atpB* mRNA as an internal standard, we did not detect any difference in the abundance of *petD* mRNA in iniD2 *vs.* S30 (Figure 3B). In other experiments we confirmed that as we have previously shown, iniD2 and S30 have a slight reduction of *petD* mRNA relative to wild-type cells (data not shown; CHEN *et al.* 1993). This suggests that *sim30-1d* does not increase the accumulation of subunit IV by increasing *petD* mRNA abundance.

We determined the subunit IV synthesis rates in wild-type, iniD2 and S30 cells by labeling chloroplast proteins with ^{14}C-acetate for 5 min in the presence of cycloheximide. Cycloheximide inhibits cytosolic protein synthesis and allows subunit IV to be visualized directly in lysates of whole cells. In such an experiment, the labeling intensity of proteins reflects their translation rates as long as the half-life of the protein investigated is very long compared to the labeling time. Figure 4 shows that the amount of subunit IV labeled in 5 min in S30 was greater than the amount labeled in the iniD2 cells. By quantifying the labeled subunit IV using the other major labeled products in the gel as internal standards, we determined that the synthesis rate of subunit IV in S30 was 50% of the WT level, which corresponds well to its abundance as measured by immunoblots (Figure 3A). We were unable to measure the iniD2 subunit IV synthesis rate accurately in this experiment because of the low amount of radioactivity incorporated into subunit IV. However, we have previously shown that in iniD2, subunit IV is synthesized at 10% of the wild-type level during a 45 min labeling period (CHEN *et al.* 1993). Therefore, the fivefold increase in the translation rate of subunit IV in S30 appears to account fully for the increase in its accumulation. Other minor differences were also reproducibly seen in the protein synthesis pattern of S30 when compared to that of iniD2. These differences may reflect altered expression of unknown chloroplast genes in S30 and could be responsible for the slightly premature sensescence of S30 cells that is discussed below.

To test the possibility that *sim30-1d* had a posttranslational effect, we performed pulse-chase experiments to measure the half-life of subunit IV in WT and S30 cells. The measured half-life was at least 4 hr in both strains, and no significant difference was observed between them (data not shown). The long half-life of subunit IV relative to the 5-min labeling period indicates that the incorporation of radioactivity into subunit IV accurately reflects its synthesis rate. We conclude that the primary effect of *sim30-1d* is to increase the accumulation of subunit IV by increasing the translation rate of *petD* mRNA with an AUU initiation codon.

***sim30-1d* is a dominant allele at a nuclear locus:** In principle, the *SIM30* gene (the wild-type allele of *sim30-1d*) could be located in the nuclear, chloroplast, or mitochondrial genome. To determine the location of *sim30-1d*, we crossed a mt^+, *sim30-1d* strain containing the *petD-AUU* mutation in the chloroplast genome (strain S30-6c, see Table 1) to a mt^- tester strain that also contained the *petD-AUU* mutation in the chloroplast genome (strain iniD2-1a). In Chlamydomonas genetic crosses, the chloroplast genome is inherited from the mt^+ parent in 95% of progeny, whereas the mitochondrial genome is normally inherited from the mt^- parent (HARRIS 1989). The accumulation of subunit IV in four complete tetrads was determined as shown in Figure 5A, using a crude antiserum raised against subunit IV. The unknown protein indicated by the arrow, which is recognized by the crude serum, serves as a convenient loading control. The progeny from all four tetrads showed a 2:2 segregation of high and low levels of subunit IV, suggesting that *sim30-1d* is an allele at a nuclear locus.

To determine whether the *sim30-1d* allele is dominant or recessive, we created diploid strains. Since only a small fraction of the zygotes formed by mating two strains give rise to vegetative diploids, diploids were selected using complementing mutant alleles of the argininosuccinate lyase gene (*arg7-8* and *arg7:* HARRIS 1989) by growing mated cells on medium lacking arginine. We crossed the *arg7* marker into iniD2 (mt^+ [*D-AUU spc*^{r}]) to create strains iniD2-1a (mt^-) and iniD2-1b (mt^+). We crossed the *arg7-8* marker into S30 and subsequently crossed an *arg7-8* progeny, S30-6c (mt^+ *arg7-8 sim30-1d* [*D-AUU spc*^{r}]), to iniD2-1a to produce diploid colonies of the S30-2n series, which have the genotype *sim30-1d/SIM30* [*D-AUU spc*^{r}]. To create con-

TABLE 1

***C. reinhardtii* strains**

Strains	Genotype[a]	Source
P17	*mt*$^+$ [+]	STERN *et al.* (1991)
iniD1	*mt*$^+$ [*D-AUC spc*^{r}]	CHEN *et al.* (1993)
iniD2	*mt*$^+$ [*D-AUU spc*^{r}]	CHEN *et al.* (1993)
A-AUU	*mt*$^+$ [*A-AUU spc*^{r}]	CHEN *et al.* (1995)
A-ACG	*mt*$^+$ [*A-ACG spc*^{r}]	CHEN *et al.* (1995)
A-ACC	*mt*$^+$ [*A-ACC spc*^{r}]	CHEN *et al.* (1995)
S30	*mt*$^+$ *sim30-1d* [*D-AUU spc*^{r}]	This research
WT-se	*mt*$^+$ [*spc*^{r} *str*^{r} *ery*^{r}]	This research
S30-wtse	*mt*$^+$ *sim30-1d* [*spc*^{r} *str*^{r} *ery*^{r}]	This research
S30-se	*mt*$^+$ *sim30-1d* [*D-AUU spc*^{r} *str*^{r} *ery*^{r}]	This research
iniD2-se	*mt*$^+$ [*D-AUU spc*^{r} *str*^{r} *ery*^{r}]	This research
CC1861	*mt*$^-$ *arg7* [+]	Chlamydomonas Genetic Stock Center
CC1930	*mt*$^-$ *arg7-8* [+]	Chlamydomonas Genetic Stock Center
iniD2-1a	*mt*$^-$ *arg7* [*D-AUU spc*^{r}]	iniD2 × CC1861 progeny
iniD2-1b	*mt*$^+$ *arg7* [*D-AUU spc*^{r}]	iniD2 × CC1861 progeny
S30-6c	*mt*$^+$ *sim30-1d arg7-8* [*D-AUU spc*^{r}]	S30 × CC1930 progeny
S30-5c	*mt*$^-$ *arg*$^-$ *sim30-1d* [*D-AUU spc*^{r}]	S30-6c × iniD2-1a progeny
WT-2n	*arg7-8/arg7* [*spc*^{r}]	Diploid from iniD2-1b × CC1930
iniD2-2n	*arg7-8/arg7* [*D-AUU spc*^{r}]	Diploid from iniD2-1b × CC1930
S30-2n	*sim30-1d/SIM30 arg7-8/arg7* [*D-AUU spc*^{r}]	Diploid from S30-6c × iniD2-1a

[a] Nuclear genotypes are followed by chloroplast genotypes, which are enclosed in brackets. For diploids, the two alleles at a nuclear locus are separated by a forward slash (/). + represents the wild-type allele; *mt*$^+$ and *mt*$^-$ are mating-type alleles; *arg7-8* and *arg7* are two mutant alleles of the arginisosuccinate lyase gene. *D-AUC* and *D-AUU* represent AUG to AUC or AUU mutations in the *petD* initiation codon; *A-AUU, A-ACG* or *A-ACC* represent AUG to AUU, ACG or ACC mutations in the *petA* initiation codon. *spc*^{r}, *str*^{r}, and *ery*^{r} represent mutations *spr-u-1-6-2, sr-u-2-60* (nucleotides 1123 and 474 in 16S rRNA) and *er-u-37* (nucleotide 2067 in 23S rRNA), which confer resistance to spectinomycin, streptomycin or erythromycin, respectively (HARRIS *et al.* 1989). Only relevant genotypes are shown in the figures.

trol diploids with two *SIM30* alleles, iniD2-1b was crossed with CC1930 (*mt*$^-$ *arg7-8*). Diploids were screened by PCR amplification and *Ssp*I digestion to determine whether they contained the *petD-AUU* mutation (see Figure 2). Because both parents contribute initially to the chloroplast genomes of diploids, heteroplasmic diploids were streaked for single colonies. Homoplasmic diploids containing either the wild-type (WT-2n) or *petD-AUU* (iniD2-2n) chloroplast genome were obtained. The accumulation of subunit IV in 14 independently isolated S30-2n strains, WT-2n and iniD2-2n was determined by immunoblotting, as shown in Figure 5B. The accumulation of subunit IV in iniD2-2n was much lower than that in WT-2n, suggesting that the *petD-AUU* mutation has the same effect on the expression of the *petD* gene in diploids as it does in haploids. The accumulation of subunit IV in all of the S30-2n strains was much increased compared to that in the iniD2-2n strain and comparable to that in S30, suggesting that the *sim30-1d* allele is dominant or semi-dominant.

***sim30-1d* suppresses the *petD-AUC* mutation:** To investigate whether the specificity of *sim30-1d* suppression is limited to the AUU mutant codon in the *petD* gene, we tested the effect of *sim30-1d* on the expression of a *petD* gene carrying an AUC mutation at the initiation codon position. To do so, we first isolated a *mt*$^-$ strain that contains the *sim30-1d* mutation from progeny of a cross between S30-6c and iniD2-1a. This strain, called S30-5c, was crossed to the *mt*$^+$ *petD-AUC*-containing strain (iniD1), which had been generated previously by chloroplast transformation. iniD1 accumulates ~10% of the wild-type level of subunit IV when grown in HSA medium at 24° (CHEN *et al.* 1993). All progeny from this cross contained the *petD-AUC* mutation as determined by PCR amplification followed by restriction enzyme digestion (data not shown). The accumulation of subunit IV in these progeny was determined by immunoblotting, as shown in Figure 6. A 2:2 segregation of high and low levels of subunit IV was observed in each tetrad. The high level was equivalent to that observed in S30, while the low level was similar to that in iniD1. These results suggest that *sim30-1d* is able to suppress the *petD-AUC* mutation, and therefore that the effect of *sim30-1d* is not initiation codon-specific.

***sim30-1d* suppresses *petA* initiation codon mutations:** To determine whether *sim30-1d*-mediated translational enhancement is restricted to the *petD* gene, we generated strains carrying *sim30-1d* in the nucleus and *petA* initiation codon mutations in the chloroplast. We have previously created strains carrying AUU, ACG or ACC mutations at the initiation codon position of the

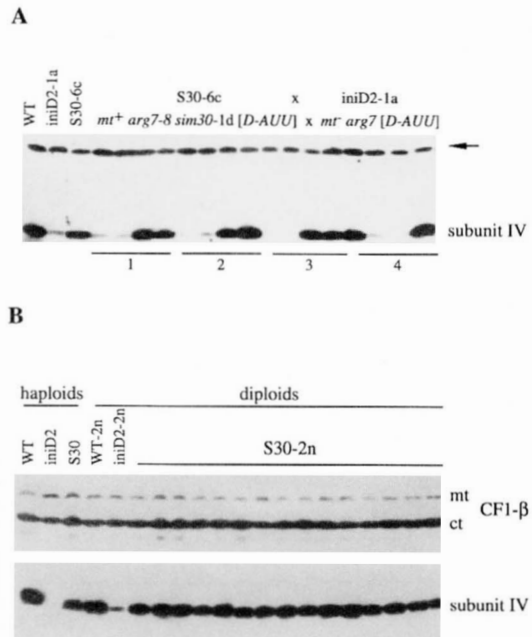

FIGURE 5.—*sim30-1d* is a dominant nuclear suppressor. (A) Subunit IV accumulation in parental strains and tetrad progeny. The lines at the bottom of the figure show groups of four tetrad progeny. The arrow on the right indicates a protein that cross-reacts with the crude serum raised against subunit IV. Although the identity of the protein is unknown, it serves as a good loading control based on its correspondence with CF1-β levels in numerous experiments. (B) Subunit IV accumulation in various haploid and diploid strains. See the text and Table 1 for genotypes. The 14 S30−2n diploids are independent isolates.

chloroplast *petA* gene. These *petA* initiation codon mutants, called A-AUU, A-ACG and A-ACC, synthesize and accumulate ~10%, 2% and 1% of the wild-type level of cytochrome f, respectively (CHEN *et al.* 1995). We crossed S30-5c (*mt*$^-$ *sim30-1d* [*D-AUU spc*^{r}]) to each of the three *mt*$^+$ *petA* initiation codon mutants and analyzed the accumulation of cytochrome f in the progeny by immunoblotting. Two tetrads from the cross involving A-AUU are shown in Figure 7, to illustrate the levels of cytochrome f expression in progeny containing *sim30-1d*. *sim30-1d* caused the accumulation of cytochrome f from the *petA-AUU* gene to increase from 10 to 50% of the wild-type level, although some variability was seen among the progeny. When progeny from the

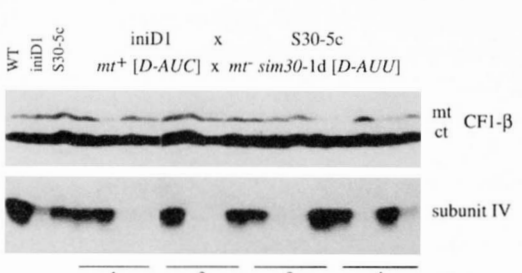

FIGURE 6.—*sim30-1d* suppresses the *D-AUC* mutation. Subunit IV accumulation was detected on immunoblots of proteins from the indicated strains and tetrad progeny.

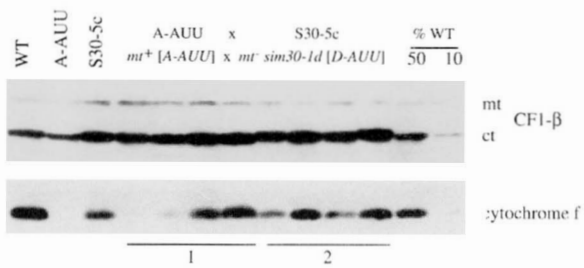

FIGURE 7.—*sim30-1d* suppresses the *A-AUU* initiation codon mutation. Cytochrome f accumulation was detected on immunoblots of proteins from the indicated strains and tetrad progeny.

crosses with A-ACG and A-ACC were examined, segregation was again seen for the accumulation of cytochrome f (data not shown). In these cases, however, the initial levels of cytochrome f were quite low (2 and 1% of WT, respectively), and it was difficult to quantify the levels in the progeny. Thus, although our data suggest that *sim30-1d* can increase cytochrome f accumulation from several different mutant initiation codons, the amount of the increase was somewhat variable, and we cannot rule out contributions of other nuclear genes.

The increase in protein accumulation caused by the *sim30-1d* suppressor mutation in the three cases carefully quantified (petD-AUU, AUC; petA-AUU) was approximately fivefold relative to the corresponding initiation codon mutant strain in a wild-type nuclear background. Furthermore, in the other cases, the absolute increase in cytochrome f accumulation mediated by *sim30-1d* was roughly correlated with the relative strength of the mutant initiation codons, so that progeny containing A-ACC or A-ACU and *sim30-1d* accumulated significantly less cytochrome f than progeny containing A-AUU and *sim30-1d*. Therefore, we conclude that the mutant *SIM30* product probably increases translation from these mutant codons, rather than acting as a bypass suppressor that causes translation to initiate at another in-frame codon.

***sim30-1d* does not affect the expression of a wild-type *petD* gene:** *sim30-1d* was isolated as a suppressor of the iniD2 mutation and appears to increase translation initiation rates of mRNAs containing non-AUG initiation codons. It was of interest to know whether the dominant *sim30-1d* allele affects the expression of a wild-type *petD* gene. We reasoned that *sim30-1d* might decrease the expression of a wild-type *petD* gene, since it is a mutant allele that accommodates non-AUG initiation codons. To address this question, we crossed S30-5c (*mt*$^-$ *sim30-1d* [*D-AUU spc*^{r}]) to P17 (*mt*$^+$ [+]). All progeny should inherit a wild-type chloroplast genome from P17, while two progeny per tetrad should contain *sim30-1d*. The accumulation of subunit IV in two tetrads from this cross is shown in Figure 8A. No difference was observed in subunit IV accumulation among the progeny, and the accumulation was equivalent to the level in wild-

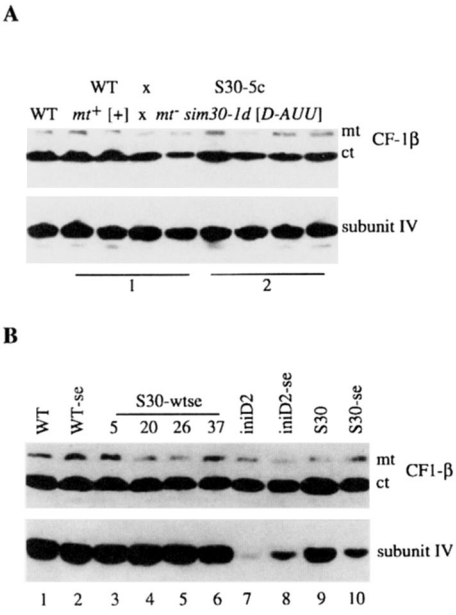

FIGURE 8.—*sim30-1d* does not affect subunit IV accumulation from a wild-type *petD* gene and may interact with the chloroplast ribosome. (A) Strains carrying a wild-type *petD* gene. Immunoblot analysis was carried out with total proteins from wild-type cells and tetrad progeny as shown. (B) Strains carrying antibiotic resistance mutations. Subunit IV accumulation was measured in the indicated strains; their genotypes are discussed in the text and are listed in Table 1. se indicates that resistance markers for streptomycin and erythromycin are present. All strains are spectinomycin resistant, with the exception of WT.

type cells, suggesting that *sim30-1d* does not significantly affect expression of the wild-type *petD* gene.

To confirm this result independently, we changed the initiation codon of the *petD* gene in S30 from AUU to AUG by chloroplast transformation. Because a spectinomycin-resistance marker was used to create the S30 progenitor strain iniD2, we utilized rDNA mutations in the plasmid p-183 that confer streptomycin and erythromycin resistance to select for chloroplast transformants of S30. We bombarded S30 with both p-183 and plasmid pBam7, which contains the wild-type *petD* gene. Transformants were selected for streptomycin resistance and then screened for the presence of the wild-type *petD* gene, as evidenced by the lack of the *Ssp*I site created by the AUU mutation (see Figure 2; data not shown). Four independent transformants carrying a wild-type *petD* gene were obtained and designated as the S30-wtse series. Each of these transformants was also found to be erythromycin resistant. We also transformed wild-type strain P17 with p-183 to create the strain WT-se, as a control. The immunoblots in Figure 8B (lanes 1–6) show that no difference was detected in subunit IV accumulation among WT, WT carrying the multiple drug resistance markers (WT-se), and S30-wtse. These results agree with those obtained independently by genetic crosses (Figure 8A) and suggest that *sim30-1d* has no adverse effects on the expression of the wild-type *petD* gene, relative to other chloroplast proteins, under the growth conditions used. We also carried out 5 min pulse-labeling experiments to measure the synthesis rate of subunit IV in WT-se and S30-wtse, and no difference in subunit IV labeling was detected (data not shown). This suggests that *sim30-1d* does not increase subunit IV synthesis from the wild-type *petD* gene relative to other chloroplast proteins. We did note, however, that WT-se was consistently and significantly greener than S30-wtse after ~10 days growth on HS plates under medium intensity light (data not shown), suggesting that *sim30-1d* may adversely affect chloroplast gene expression or cell growth in ways that we have not detected.

Although the multiple drug resistance markers had no effect on the expression of subunit IV from a wild-type *petD* gene, this was not the case when the *petD*-AUU initiation codon mutation was combined with *sim30-1d*. We created the strain S30-se (*mt*$^+$ *sim30-1d* [*D-AUU spc*^{r} *str*^{r} *ery*^{r}]) by transforming S30 with p-183. Figure 8B (lanes 9 and 10) shows that subunit IV accumulation in S30-se was at least twofold lower than in S30, after normalizing to CF1-β. We speculate that the str^r and/or ery^r markers (in 16S and 23S rRNA, respectively) may affect the ability of *sim30-1d* to increase subunit IV synthesis from the *petD-AUU* transcript, or alternatively, that the mutation(s) decrease the synthesis of subunit IV from the *petD-AUU* transcript independently of *sim30-1d*. To test the second possibility, we created the strain iniD2-se ([*D-AUU spc*^{r} *str*^{r} *ery*^{r}]) by transforming iniD2 ([*D-AUU spc*^{r}]) with p-183. For reasons that are unclear, the accumulation of subunit IV in iniD2-se was approximately twofold higher than that in iniD2 (Figure 8B, lanes 7 and 8). However, this does demonstrate that the rRNA mutations do not adversely affect expression of the *petD-AUU* gene. Therefore, we conclude that one or both rRNA mutations interfere with the ability of *sim30-1d* to suppress the iniD2 defect. The mechanistic implications of this finding are discussed below.

DISCUSSION

We have shown that *sim30-1d* is a dominant nuclear allele, and that it increases the translation rate of *petD-AUU* mRNA. Although *sim30-1d* was originally isolated as a suppressor of the *petD-AUU* mutation, it was found to suppress the *petD-AUC* mutation and an AUU mutation in the *petA* initiation codon. We therefore conclude that its action is neither codon-specific nor gene-specific. These properties strongly suggest that *sim30-1d* either encodes or regulates the synthesis or activity of a general chloroplast translation factor. Because most of the proteinaceous components of the chloroplast translational apparatus are encoded in the nucleus, it would not be surprising if *SIM30* encodes a ribosomal protein or initiation factor.

Because *sim30-1d* increases protein accumulation in

proportion to the basal level for each initiation codon mutant tested, it probably increases translation efficiency from the mutant codon, rather than acting as a bypass suppressor that allows translation initiation at an alternative codon. In the latter case, each mutant codon, when suppressed by *sim30-1d,* would be expected to accumulate the same amount of cytochrome f.

The likely mode of action of the *sim30-1d* product differs from other examples of initiation codon mutation suppressors. In yeast, three suppressors of an AUG to AUU mutation in the nuclear *HIS4* gene have been isolated. These suppressors identified the *sui1, sui2* and *SUI3* genes, which when mutated, activated translation initiation at a UUG triplet two codons downstream of the original initiation codon. Thus the *sui* gene products, two of which were found to be components of eIF-2, alter the cytoplasmic ribosomal scanning process (DONAHUE *et al.* 1988; CIGAN *et al.* 1989; CASTILHO-VALAVICIUS *et al.* 1990; YOON and DONAHUE 1992).

Two other types of suppressors were isolated for yeast mitochondrial initiation codon mutants. A cold-sensitive suppressor of a *COX3* AUG to AUA mutation was found to inactivate one of two genes encoding the cytoplasmic small ribosomal subunit protein S18 (FOLLEY and FOX 1994). This suppression mechanism appears to be indirect, since S18 cannot be detected in mitochondria. In contrast, a *COX2* AUG to AUU mutation was partially suppressed by increased dosage of the gene-specific mitochondrial translation factor coded by the nuclear *PET111* gene (MULERO and FOX 1994). Although the Chlamydomonas *sim30-1d* product most likely acts directly in the chloroplast translation initiation process, it is certainly possible that bypass and other types of suppressors may also be identified for chloroplast translation initiation mutants.

Our (CHEN *et al.* 1993, 1995) and others' (BETTS and SPREMULLI 1994; KIM and MULLET 1994; MAYFIELD *et al.* 1994) analyses of chloroplast translation initiation suggest that for at least some chloroplast mRNAs, the 30S ribosomal subunit binds internally as it does in *E. coli.* The initiation of protein synthesis in *E. coli* involves a series of reactions that result in the formation of the 70S initiation complex in which the 30S and 50S subunits are associated with each other and with the mRNA, such that the fMet-tRNA is located in the P site on the ribosome. The rate-limiting step in *E. coli* translation initiation is the formation of the 30S initiation complex, which is composed of a 30S ribosomal subunit, mRNA, fMet-tRNA, GTP, and initiation factors IF-1, IF-2 and IF-3 (reviewed in MCCARTHY and GUALERZI 1990).

Because the *ery*^{r} and/or *str*^{r} rRNA mutations impair the ability of *sim30-1d* to suppress the *petD-AUU* mutation, it is possible that the *sim30-1d* gene product interacts with one of the ribosomal subunits. Within the context of a prokaryotic model for chloroplast translation initiation, potential mechanisms of action for the mutant *sim30-1d* protein can be proposed. If the *ery*^{r} 23S rRNA mutation interferes with the action of *sim30-1d* (see Figure 8B), we would propose that the *sim30-1d* product most likely functions after the formation of the 30S initiation complex, at the level of 50S ribosomal subunit binding. The *sim30-1d* product may alter the equilibrium of the 30S to 70S transition by promoting the 50S-30S interaction, thus depleting the pool of 30S complexes and driving the formation of the 30S initiation complex. For wild-type mRNAs, 30S initiation complexes may be sufficiently abundant that the *sim30-1d* product has no net effect on the protein synthesis rate. The product of the *SIM30* gene might be a component of either ribosomal subunit, or a peripheral ribosomal protein, such as the *SUI1* product discussed above. Alternatively, the *SIM30* product may regulate the synthesis or activity of such a protein.

On the other hand, if the 16S rRNA *str*^{r} mutation interferes with the action of the *sim30-1d* product, we would propose that the *sim30-1d* product is directly or indirectly involved in the assembly of the 30S initiation complex. The *str*^{r} mutation is located within a single-stranded region, the Chlamydomonas counterpart of the *E. coli* 530 loop in 16S rRNA (DRON *et al.* 1982). The 530 loop has been implicated in the modulation of translation initiation efficiency in *E. coli* by interacting with nucleotides upstream of the initiation codon (LAGUNEZ-OTERO 1993). Mutational analysis of the nucleotides surrounding the *petA* initiation codon raised the possibility that the nucleotides in the Chlamydomonas 530 loop may also modulate translation initiation efficiency (CHEN *et al.* 1995). We would speculate that the *sim30-1d* product may modulate the interaction between the 530 loop and the initiation codon region of the mRNA.

A final possibility for the identity of *SIM30* is that this gene is a homologue of *infC* that encodes IF-3. In *E. coli, infC* has an AUU initiation codon that is involved in negative feedback regulation of its synthesis. Certain mutations in *infC* can overcome this feedback regulation, thus augmenting the production of IF-3 (SACERDOT *et al.* 1996; SUSSMAN *et al.* 1996). However, this effect was neither specific to *infC* nor to AUU: reporter genes containing a "rare" initiation codon (other than AUG, GUG, or UUG) showed increased expression in the various *infC* mutant backgrounds. Furthermore, the three- to fivefold increase in reporter gene expression was proportional to their basal level of expression in a wild-type *infC* background. If *sim30-1d* did represent a mutation in a Chlamydomonas nuclear *infC* gene, however, this would not explain its interaction with rRNA mutations, except by an indirect mechanism.

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